Biosynthesis of α -Farnesene and its Relation to Superficial Scald Development in 'Delicious' Apples

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ABSTRACT. α -Farnesene is an acyclic sesquiterpene hydrocarbon that is a constituent of the volatile components and the surface wax of apples (*Malus × domestica* Borkh.). Although oxidation products of α -farnesene have been implicated in the development of superficial scald in apples, the relation between α -farnesene biosynthesis and scald development is not well understood. In vivo labeling studies using isolated tissue segments showed that α -farnesene is derived from *trans,trans*-[1,2-¹⁴C or 1-³H]-farnesyl pyrophosphate (FPP) mostly in the skin rather than cortex tissue. Among other labeled products, farnesol was >100-fold higher compared to α -farnesene. However, HPLC analysis of hexane-extractable components from apple skin revealed farnesol is not a predominant natural constituent of apple skin tissue. In addition, *trans,trans*-[1-³H]-farnesol was not converted to α -farnesene by apple skin tissue. Our results indicate that biosynthesis of α -farnesene in apple tissue occurs through the isoprenoid pathway, and the conversion of FPP to α -farnesene is catalyzed by a single sesquiterpene synthase enzyme, *trans,trans*- α -farnesene synthase, rather than via farnesol as an intermediate. A comparison of α -farnesene biosynthesis between scald-developing and scald-free regions of the same apple showed that incorporation of radiolabel into α -farnesene from *trans,trans*-[1-³H]-FPP was nearly 3-fold lower in scald-developing skin tissue than in scald-free skin tissue.

Superficial scald is a serious storage disorder of several apple cultivars, characterized by brown or bronze discoloration of the skin (Bain and Mercer, 1963; Meigh, 1969). α -Farnesene, an acyclic sesquiterpene hydrocarbon (C₁₅H₂₄; [3*E*,6*E*]-3,7,11-trimethyl-1,3,6,10-dodecatetraene), which is a constituent of the surface wax of apples, has long been associated with the occurrence of this physiological disorder (Huelin and Coggiola, 1970a, 1970b). Oxidation products of α -farnesene, including hydroperoxides or conjugated trienes, are presumed to be the potential causative factors for the development of scald (Anet, 1972). Conjugated trienes progressively accumulate on the surface of apples during storage. The concentration of trienes, as measured by UV spectroscopy, correlates more closely with the occurrence and severity of superficial scald than does the concentration of α -farnesene (Chen et al., 1990; Gallerani and Pratella, 1991; Huelin and Coggiola, 1970b; Meir and Bramlage, 1988). In the natural coating of apple, the α isomer of farnesene was isolated and identified as the predominant form (Anet, 1970; Huelin and Murray, 1966; Murray et al., 1964; Naves, 1966). Subsequently, Murray (1969) confirmed that *trans,trans*- α -farnesene from apples is predominant over the *cis,trans* isomer in a 300:1 ratio. Based on the analyses of the triterpene (C₃₀) acids (mainly ursolic and oleanolic) in the natural coating of apples, Murray (1969) suggested that α -farnesene might originate from a farnesyl or a nerolidyl intermediate. Recently, Salin et al. (1995) reported that *trans*- β -farnesene in maritime pine (*Pinus pinaster* Ait.) needles was biosynthesized from *trans,trans*-farnesyl pyrophosphate (FPP). As well, there are many reports on the biosynthesis of sesquiterpene hydrocarbons from *trans,trans*-FPP (see Croteau and Cane, 1985).

Presently, superficial scald is controlled commercially by post-harvest treatment of apples with the antioxidant diphenylamine (DPA) (Anet and Coggiola, 1974; Huelin and Coggiola, 1970a, 1970b). Because of increasing concern over potential undesirable effects of DPA residues or metabolites to the consumer, development of alternative nonchemical control measures are increasingly being sought (Ingle and D'Souza, 1989; Lurie et al., 1991). A better understanding of the biochemical basis of scald development will help in the design of nonchemical control strategies such as a preharvest prediction method. Hence, the present study was undertaken to understand the biochemical mechanism of α -farnesene biosynthesis in 'Delicious' apples that develop severe scald during storage.

Materials and Methods

PLANT MATERIAL. 'Delicious' apples were harvested at optimum maturity during the first week of October 1996 from a commercial grower in Meaford, Ontario, and were transported to Guelph. Apples were cooled to 0 °C within 8 h of harvest and kept at 0 °C in air or in controlled atmosphere (CA) of 3 kPa O₂ and 2.5 kPa CO₂.

CHEMICAL REAGENTS. *Trans,trans*-[1-³H]-farnesyl pyrophosphate (7.4 × 10¹⁴ Bq·mol⁻¹), *trans,trans*-[1,2-¹⁴C]-farnesyl pyrophosphate (2.0 × 10⁹ Bq·mol⁻¹), *trans,trans*-[1-³H]-farnesol (2.1 × 10¹⁵ Bq·mol⁻¹), [5-³H]-mevalonate (2.2 × 10¹⁵ Bq·mol⁻¹), and [2-¹⁴C]-acetic acid sodium salt (2.0 × 10¹² Bq·mol⁻¹) were purchased from American Radiolabeled Chemicals (ARC) Inc., St. Louis, Mo. A standard mixture of α , β isomers of farnesene was obtained from TCI, Tokyo; standards of farnesol and farnesyl acetate were from Aldrich Chem. Co., Milwaukee, Wis.; and NADP and ATP were from Boehringer Mannheim Canada Inc., Montreal, Que. All other reagents were from Sigma-Aldrich Canada Ltd., Oakville, Ont.

FRUIT TISSUE PREPARATION. Apples stored in CA for 5 months (if not otherwise mentioned) were used in these experiments. Fruit were brought to room temperature the day before being used in the experiment. Fruit showing initial signs of scald on only one side were selected for these experiments such that they could be cut (axial plane) into scalding and scald-free halves. Under these

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experimental conditions, we observed this specific cultivar, when left at room temperature, develops scald only on one side. This provided the added advantage of eliminating experimental errors due to physiological and biochemical differences among fruit. Cylindrical pieces of tissue were removed from the equatorial regions of scalding or scald-free halves using a cork borer (1 cm diameter), and divided into skin (2 mm thick; 104 ± 12 mg fresh mass), and outer (closer to the skin) and inner (closer to the core) cortex segments (5 mm thick; 273 ± 17 mg fresh mass).

CELL-FREE EXTRACT PREPARATION. Outer regions of apple tissue (0.5 to 0.75 cm) were removed and cut into 0.5 cm³ pieces and homogenized using a Polytron homogenizer (model PT 10/35; Brinkmann Instruments, Westbury, N.Y.) for 1 min in sodium phosphate buffer (0.1 mol·L⁻¹, pH 6.5) containing 5 mol·L⁻¹ sucrose, 50 mmol·L⁻¹ ascorbic acid, 2 mmol·L⁻¹ EDTA, 1 mmol·L⁻¹ DTT, 1 mmol·L⁻¹ PMSF, and 50 g·L⁻¹ polyvinylpyrrolidone. The homogenate was filtered through four layers of cheesecloth, the filtrate was centrifuged at 750 g_n for 10 min to remove starch and debris, and the supernatant was used as the cell-free extract. Protein content of the extract was determined by the method of Bradford (1976).

IN VIVO INCORPORATION OF RADIOLABELLED PRECURSORS. Fruit tissue segments, prepared as described above, were incubated in 4.5 mL amber glass vials containing 950 µL of 0.1 mol·L⁻¹ phosphate buffer (pH 7.2). The vials were sealed tightly using screw caps with Teflon-silicon septa. Air trapped in the vial was removed using a needle and tubing connected to a vacuum pump and flushed with pure N₂ (99.995 kPa). The reaction was started by adding 50 µL of radiolabelled substrate (equivalent to ≈25 pmol substrate; 1.85×10^5 Bq of radiolabelled precursor in 1 mL final volume of 0.1 mol·L⁻¹ phosphate buffer and 1 mL·L⁻¹ Triton X-100) to the reaction vial containing tissue segments. The tissue segments were subjected to vacuum infiltration to facilitate the uptake of the radiolabelled substrate and filled again with pure N₂ to minimize the possible oxidative breakdown of the product, α-farnesene. After 1 h of incubation with gentle shaking, the reaction was terminated by adding 0.1 mL of 1 mol·L⁻¹ KOH and 1 mL hexane followed by vigorous mixing. A randomized complete-block design with three fruit (blocks) was used for all experiments. For time-course comparison of α-farnesene synthesis in scalding versus scald-free tissue, each block consisted of scalding and scald-free tissues each with four reaction times. Two experiments were conducted independently and data were pooled for statistical analysis (SAS release 6.12, SAS Institute, Cary, N.C.).

SEPARATION AND ANALYSIS OF RADIOLABELLED PRODUCTS. One-half milliliter of hexane extract was transferred to a test tube that contained a mixture of farnesene, farnesol and farnesyl acetate standards, and evaporated to near dryness under N₂. The remaining residue was redissolved in 50 µL of hexane and 25-µL aliquots were spotted on a thin layer chromatography plate (silica gel G, 60

× 10⁻¹⁰ m, 20 × 20 cm, Whatman Inc., Clifton, N.J.). The plates were completely developed with 4 hexane : 1 ethyl ether (v/v) and dried under N₂. α-Farnesene ($R_f = 0.659$), farnesyl acetate ($R_f = 0.505$), and farnesol ($R_f = 0.203$) were visualized in the presence of iodine vapor. The regions of the plate corresponding to authentic standards were scraped, mixed with 5 mL of scintillation cocktail (Ecolume, ICN, Costa Mesa, Calif.), and their radioactivity determined by liquid scintillation counting (Beckman LS6800, Mississauga, Ont.). Controls, of no tissue segment and boiled tissue segment, were used to exclude the possible formation of compounds by nonenzymatic reaction(s). Nonenzymatic formation of α-farnesene and farnesyl esters was negligible. The 8% of total radioactivity that accumulated in the farnesol fraction was due to nonenzymatic conversion of the substrate.

ANALYSIS OF α-FARNESENE CONTENT USING HPLC. Two segments of fruit skin tissue, prepared as described above, were immersed in 2 mL of hexane, and kept for 24 h in a closed 4.5-mL glass vial. Hexane-soluble α-farnesene content was analyzed by HPLC by measuring absorbance at 233 nm after separation on a Nova-Pak C₁₈ column (3.9 × 150 mm, 60 × 10⁻¹⁰ m, Waters Co. Milford, Mass.). Acetonitrile (100%) was used as the mobile phase, at a flow rate of 0.75 mL·min⁻¹. Farnesol was eluted at 2.3 min, and farnesene at 2.8 min. Two experiments were conducted independently and data were pooled for statistical analysis (SAS release 6.12).

Results and Discussion

TISSUE LOCALIZATION AND α-FARNESENE BIOSYNTHESIS. Experiments using isolated tissue segments showed that incorporation of radiolabel from *trans,trans*-[1-³H]-farnesyl pyrophosphate (FPP) into α-farnesene was limited to the skin tissue when compared to the cortex tissues (Table 1). Similar results were also obtained while using *trans,trans*-[1,2-¹⁴C]-FPP as substrate (data not presented). This provides direct evidence for the conversion of FPP into α-farnesene by apple skin tissue. Scald symptoms are confined to the hypodermal and epidermal cell layers of the fruit (Fidler, 1950). Histological investigations by Bain (1956) and Bain and Mercer (1963) also revealed that the browning of cells was limited to the epidermal layer and the first five or six layers of the hypodermis. Hence, it can be speculated that all biochemical changes that cause scald development, including the biosynthesis of α-farnesene, could be localized in the few outer cell layers of apples. At harvest, α-farnesene formation from [1-³H]-FPP was very low (6 Bq·g⁻¹·h⁻¹), but increased after 3 and 20 weeks in air and CA, respectively, and then declined after 28 weeks in CA storage (Table 1).

Among the other labeled products detected in the hexane extract, farnesol was predominant but there was also a trace

Table 1. Incorporation of *trans,trans*-[1-³H]-farnesyl pyrophosphate into α-farnesene and farnesol by different tissue segments of 'Delicious' apples.

Tissue segment	Storage time in air or CA ² at 0 °C					
	Air, 3 weeks		CA, 20 weeks		CA, 28 weeks	
	FNE ^y	FOL ^x	FNE	FOL	FNE	FOL
Radioactivity on a fresh mass basis (Bq·g ⁻¹ ·h ⁻¹)						
Skin	26	4810	73	2075	40	2046
Outer cortex	0	4904	0	1942	0	1643
Inner cortex	0	5280	0	2092	0	1719

²CA = controlled atmosphere storage of 3.0 kPa O₂ and 2.5 kPa CO₂ at 0 °C.

^yFNE = α-farnesene, standard error of mean for FNE = 14.

^xFOL = farnesol, standard error of mean for FOL = 130.

Fig. 1. Proposed pathway and mechanism of α -farnesene biosynthesis in apple skin tissue. HMG CoA = 3-hydroxy-3-methylglutaryl-CoA, HMGR = HMG CoA reductase, IPP = isopentenyl pyrophosphate, DMAPP = dimethylallyl pyrophosphate, GPP = geranyl pyrophosphate.

amount of farnesyl acetate. The formation of farnesol from $[1-^3\text{H}]$ -FPP was >100-fold higher than α -farnesene formation in fruit three weeks after harvest. Farnesol formation in the skin, and in the outer and inner cortex tissue segments was similar (Table 1). Formation of farnesol from FPP by the action of phosphatases has been reported in other plant tissue systems (Croteau and Karp, 1979; Dehal and Croteau, 1988; Vögeli and Chappel, 1988) and could account for the large amounts of farnesol relative to α -farnesene formed from FPP in apple tissue. Thus, the possibility exists that farnesol may be an intermediate in the formation of α -farnesene from FPP.

Trans,trans-farnesol is the only possible isomer that could be formed from *trans,trans*-FPP, and it is the only stereoisomer present in many essential oils (Naves, 1966). However, *trans,trans*- $[1-^3\text{H}]$ -farnesol was not converted to α -farnesene either in isolated tissue segments or in a cell-free system, although loss of radioactivity from labeled farnesol was observed both in vivo and in vitro. In a cell-free system, labeled farnesol decreased with time in the presence of increasing Mg^{2+} concentration without formation of α -farnesene (data not presented), suggesting that farnesol may be metabolized into other products. The capability of phosphorylating farnesol in the presence of Mg^{2+} and cytidine triphosphate (CTP) to give the corresponding mono- (FP) and diphosphate (FPP) through the action of farnesol (pyro)phosphokinase has been reported in the 100,000 g_n pellet fraction of *Botryococcus braunii* Kützing Brace (Inoue et al., 1995). However, α -farnesene

cannot be formed readily by dehydration of the sesquiterpene alcohols, farnesol or nerolidol (Murray, 1969). As well, dehydration of farnesol can yield only the *trans*- β -farnesene isomer (Brieger et al., 1969; Naves, 1966).

Labeled precursors of the isoprenoid pathway, $[2-^{14}\text{C}]$ -acetic acid and $[5-^3\text{H}]$ -mevalonate, were incorporated into α -farnesene in trace amounts in skin tissue segments but not in cortex tissues. In both these experiments, farnesol formation was not higher than that of α -farnesene (data not presented). However, incubation of $[2-^{14}\text{C}]$ -acetic acid with the cell-free extract in the presence of 1 mmol·L⁻¹ coenzymeA, 5 mmol·L⁻¹ ATP, 10 mmol·L⁻¹ MgCl_2 , 1 mmol·L⁻¹ NADPH, 5 mmol·L⁻¹ ascorbate, and 1 mmol·L⁻¹ DTT, or incubation of $[5-^3\text{H}]$ -mevalonate in the presence of 50 $\mu\text{mol}\cdot\text{L}^{-1}$ mevastatin (inhibitor of HMGR), 5 mmol·L⁻¹ ATP, 10 mmol·L⁻¹ MgCl_2 , 1 mmol·L⁻¹ NADPH, 5 mmol·L⁻¹ ascorbate, and 1 mmol·L⁻¹ DTT did not lead to the formation of α -farnesene. The incorporation of $[^{14}\text{C}]$ -acetate and $[^3\text{H}]$ -mevalonate into plant sesquiterpenes has been reported (Cane, 1981). The poor incorporation in the present study, however, may be due to the mobilization of the labeled precursors into several other pathways.

Our results revealed that α -farnesene biosynthesis in apple skin is mainly through a direct conversion of FPP to α -farnesene rather than via farnesol as an intermediate. α -Farnesene formation from FPP could directly occur through a deprotonation reaction of an allylic carbocation (McGarvey and Croteau, 1995), catalyzed by a sesquiterpene synthase enzyme, *trans,trans*- α -farnesene synthase (Fig. 1). Isolation of *trans,trans*- α -farnesene has also been reported in pears (*Pyrus communis* L.) (Murray, 1969), hops (*Humulus lupulus* L. var. Cordifolius Maxim.) (Naya and Kotake, 1970), dwarf Cavendish bananas (*Musa accuminata* Colla) (Wills et al., 1975), 'Tahitian' lime (*Citrus latifolia* Tanaka), 'Emperor' mandarin (*Citrus reticulata* Blanco), 'Marsh' grapefruit (*Citrus paradisi* Macf), and 'Valencia' orange (*Citrus sinensis* L Osbeck) (Moshonas and Shaw, 1980; Yuen et al., 1995). This is the first report illustrating its biosynthesis.

α -FARNESENE METABOLISM IN SCALDING AND SCALD-FREE TISSUE.

To investigate the possible relationship between α -farnesene biosynthesis and superficial scald development, α -farnesene content of the fruit skin and incorporation of radiolabel into α -farnesene from $[1-^3\text{H}]$ -FPP were analyzed using skin tissues from scald-developing and scald-free regions of the same apple. Interestingly, α -farnesene content, as estimated by HPLC separation and quan-

Fig. 2. α -Farnesene content of scald-free and scalding 'Delicious' apple skin tissue as estimated by separation of hexane-extracted components by high-performance liquid chromatography. Each bar represents the mean of six replications, and the error bar indicates the standard error of the mean.

Fig. 3. Incorporation of radioactivity into α -farnesene (A) and farnesol (B) from *trans,trans*-[1- 3 H]-FPP in scalding (●) and scald-free (■) 'Delicious' apple skin tissue during incubation. Two experiments each with three fruit were conducted independently and data were pooled for the statistical analysis.

tification by monitoring absorbance at 233 nm, was nearly three times higher in scald-free skin tissue compared with scalding skin tissue (Fig. 2). Farnesol was not detected as a predominant constituent among the hexane-extractable components of scalding or scald-free tissue. Previous studies have shown that α -farnesene emanates from the fruit surface, and hexane-extractable α -farnesene content of the skin is higher in scald-free apple tissues than scalding tissues (Paliyath et al., 1997; Whiting et al., 1997).

Labeled α -farnesene and farnesol production from [1- 3 H]-FPP increased as a function of time (Fig. 3 A and B). α -Farnesene formation from [1- 3 H]-FPP was 3-fold greater in scald-free skin tissue (Fig. 3A), showing parallelism to the natural α -farnesene levels in the skin (Fig. 2). Farnesol formation from [1- 3 H]-FPP was not significantly different ($p = 0.05$) between scalding and scald-free tissues (Fig. 3B). These results indicate that α -farnesene formation is independent of farnesol formation despite scald incidence. Accumulation of lower levels of α -farnesene and reduced α -farnesene biosynthesis in scald-developing tissue suggest that α -farnesene catabolism and/or α -farnesene biosynthesis are affected by scald development. The apparent fate of labeled α -farnesene and potential differences between the scald-developing and scald-free tissues are under investigation. The fact that hexane-extractable α -farnesene content progressively increases during the first 2 months of storage and declines thereafter is well documented (Huelin and Murray, 1966; Meigh, 1969; Meigh and Filmer, 1969; Meir and Bramlage, 1988). Therefore, it would be expected that α -farnesene synthase attains maximal activity during the first 2 to 3 months in storage and then declines, after which time the initial symptoms of scald are seen. If so, this could explain

the present results of changes in α -farnesene synthase activity during storage. In addition, HPLC provides a more precise estimation of α -farnesene level than spectrophotometric estimation of the crude skin extract, since spectrophotometric methods could over- or underestimate α -farnesene content as a result of interference of other surface components in scald-developing apples. Whiting et al. (1997) observed over 11 separate components of apple skin that absorb at 233 nm. Thus, this could be the reason for widely recognized poor correlation between concentration of α -farnesene content (measured by spectrophotometry) and the extent and severity of superficial scald reported by several laboratories (Chen et al., 1990; Gallerani and Pratella, 1991; Huelin and Coggiola, 1970b; Meir and Bramlage, 1988).

In summary, our results indicate that α -farnesene formation in apple tissue is through the isoprenoid pathway via FPP, and is localized predominantly in epidermal and hypodermal cell layers of fruit. Conversion of FPP to α -farnesene is catalyzed by a single sesquiterpene synthase, *trans,trans*- α -farnesene synthase, rather than via farnesol as an intermediate. Reduced biosynthesis of α -farnesene from [1- 3 H]-FPP, and lower content of accumulated α -farnesene in the skin of scald-developing tissue, is due either to enhanced catabolism of α -farnesene and/or to inhibition of specific enzymes of the α -farnesene biosynthetic pathway, or may simply reflect enhancement of cellular damage as scald forms.

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